Mid-trimester fetal blood-derived adherent cells share characteristics similar to mesenchymal stem cells but full-term umbilical cord blood does not

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Summary

Stem cell transplantation is a promising treatment for many conditions. Although stem cells can be isolated from many tissues, blood is the ideal source of these cells due to the ease of collection. Mesenchymal stem cells (MSCs) have been paid increased attention because of their powerful proliferation and pluripotent differentiating ability. But whether MSCs reside in blood (newborn umbilical cord blood and fetal or adult peripheral blood) is also debatable. The present study showed that MSC-like cells could be isolated and expanded from 16–26 weeks fetal blood but were not acquired efficiently from full-term infants' umbilical cord blood (UCB). Adherent cells separated from postnatal UCB were heterogeneous in cell morphology. Their proliferation capacity was limited and they were mainly $CD45^+$, which indicated their haematopoietic derivation. On the contrary, MSC-like cells shared a similar phenotype to bone marrow MSCs. They were $CD34^-$ $CD45^-$ $CD44^+$ $CD71^+$ $CD90^+$ $CD105^+$. They could be induced to differentiate into osteogenic, adipogenic and neural lineage cells. Single cell clones also showed similar phenotype and differentiation ability. Our results suggest that early fetal blood is rich in MSCs but term UCB is not.

Keywords: fetal blood, umbilical cord blood, mesenchymal stem cells, phenotype, differentiation.

Stem cell transplantation is a promising therapy for degenerative and necrotic diseases (Horwitz *et al*, 1999; McDonald *et al*, 1999; Orlic *et al*, 2001; Pluchino *et al*, 2003). There are many types of stem cells, residing in different tissues. Among them, pluripotent stem cells have been paid more attention because of their multilineage differentiation ability. Mesenchymal stem cells (MSCs) and multipotent adult progenitor cells (MAPCs) (Reyes *et al*, 2001; Jiang *et al*, 2002a) are fibroblast like cells, they possess powerful proliferation capacity and are considered as pluripotent stem cells. Pluripotent stem cells are not determined by powerful proliferation capacity. MSCs can be obtained from adult bone marrow; they comprise a small population but can be expanded exponentially under favourable conditions (Prockop, 1997; Pittenger *et al*, 1999). Some reports also suggested MSCs and MAPCs can be effectively separated from many other tissues (Campagnoli *et al*, 2001; Zuk *et al*, 2001; Arai *et al*, 2002; Jiang *et al*, 2002b; Sottile *et al*, 2002; Romanov *et al*, 2003).

Convenient collection makes blood superior to other tissues as the source of therapeutic cells. That haematopoietic stem cells reside in umbilical cord blood (UCB) and mobilized peripheral blood is universally accepted, but whether MSCs reside in blood (UCB and peripheral blood) remains in dispute. Some reports showed that UCB and adult peripheral blood lack MSCs (Hows *et al*, 1992; Ojeda-Uribe *et al*, 1993; Lazarus *et al*, 1997; Mayani *et al*, 1998; Gutierrez-Rodriguez *et al*, 2000; Mareschi *et al*, 2001; Wexler *et al*, 2003). Fernandez *et al* (1997) first reported that stromal cells could be separated from mobilized peripheral blood but could not from immobilized blood. Some reports have shown that MSCs circulate in adult human peripheral blood but this is extremely rare (Zvaifler *et al*, 2000; Kuznetsov *et al*, 2001). Erices *et al* (2000) showed that MSCs could be separated from UCB but this was mainly from preterm UCB. Goodwin *et al* (2001) showed that MSC-like cells, called non-haematopoietic progenitors, could be isolated from UCB but need more time

compared with bone marrow. Campagnoli *et al* (2001) reported that MSCs could be effectively obtained from first-trimester fetal blood, at a mean number of $8{\cdot}2 \pm 0{\cdot}6/10^6$ nucleated cells. These reports indicate that full-term infant UCB and preterm fetal blood might have different characteristics. In the present study, we executed a series of experiments and confirmed this hypothesis. We also showed that fetal blood-derived MSCs could differentiate into osteocytes, adipocytes, neurons and astrocytes, and possessed powerful proliferation capacity and similar surface markers to bone marrow MSCs (BMMSCs). Cell colonies also had similar phenotype and differentiation potentials.

Materials and methods

Cell culture and harvest

Umbilical cord blood and fetal blood samples were obtained, with the mother's consent, from the local maternity hospital. Permission to use human tissue was granted by the Ethical Committee of the Peking University Health Science Centre. Full-term UCB samples were collected at the time of birth ($n = 19$). Preterm fetal blood samples were collected from cords of fetus during pregnancy through centesis under ultrasound guidance or from the hearts and thoracic aortas of aborted fetuses ($n = 9$). Mononuclear cells (MNCs) were isolated using standard Ficoll–Hypaque techniques and then cultured in α-minimum essential medium (α-MEM; Gibco BRL, Grand Island, NY, USA) containing 15% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 2 mmol/l L-glutamine (Gibco BRL) and seeded at a concentration of 5×10^5 cells/cm^2. Cultures were maintained at 37°C in a humified atmosphere containing 5% CO_2. After culture for 48 h for the culture medium was replaced and non-adherent cells were removed. The medium was then changed every 3–4 d. After 14 d, fibroblast colony-forming units (CFU-F; per clone cell number >50) were counted. Cells in the developed adherent layer were used for the experiments. At 80% confluence, cells were harvested with 0·25% trypsin (Gibco BRL) for 5 min at 37°C and were passaged at a ratio of 1:3. Cell clones, obtained through fluorescent-activated cell sorting (FACS), were plated into 96-well plates at single cell per well and were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco BRL) containing 10% FBS and 5 ng/ml basic fibroblast growth factor (bFGF; Gibco BRL) (Tsutsumi *et al*, 2001).

Phenotype analysis using FACS

Adherent cells were trypsinized and stained with mouse monoclonal phycoerythrin (PE)-labelled antibodies anti-CD34, CD44, CD90, CD117 or fluorescein isothiocyanate (FITC)-labelled antibodies anti-CD14, CD45, CD71, CD147, HLA-DR (BD Pharmingen, San Diego, CA, USA), CD105 (Southern Biotech Associates, Inc., Birmingham, AL, USA) and Bcrp1 (Chemicon, Temecula, CA, USA). Mouse IgG_1-PE, IgG_1-FITC, IgG_{2a}-PE, IgG_{2b}-FITC (BD Pharmingen), IgG_{2a}-FITC (Chemicon), IgM-FITC (Southern Biotech Associates, Inc.) were used as isotype controls. Stained cells were analysed by FACScalibur flow cytometry (BD Pharmingen).

Cell cycle analysis and proliferation studies

For cell cycle analysis, CFU-F derived cells (CFU-FC) were permeabilized with 70% alcohol on ice, then treated with 100 μg/ml RNANaseA (Sigma-Aldrich, St Louis, MO, USA) and labelled with 20 μg/ml propidium iodide (Sigma), DNA content was assessed by FACScan using the MODFIT software (BD Pharmingen, San Diego, CA, USA).

For proliferation studies, the number of CFU-FC was counted at each passage ($n = 1$). Cell doublings were determined through cell enumeration. The proliferation rate of the clone-derived cells was determined by randomly selecting clones ($n = 11$) and clone-derived cells were enumerated at each passage.

In vitro differentiation

Osteogenic differentiation. Adherent cells were cultured in osteogenic medium (Jaiswal *et al*, 1997), which consisted of DMEM (Gibco BRL) supplemented with 10% FBS, 10 mmol/l β-glycerophosphate (Sigma), 100 nmol/l desamethasone (Jinhua Pharmaceutical Factory, Tiajin, China), 0·25 mmol/l L-ascorbic acid (Sigma) and fed daily with L-ascorbic acid. After 3 weeks of induction, cells were stained using the von Kossa procedure to assess mineralization (Zuk *et al*, 2001). Osteoblasts differentiation was evaluated by alkaline phosphatase (ALP) expression.

Adipogenic differentiation. Adherent cells were cultured in DMEM supplemented with 10% FBS, 0·5 mmol/l isobutylmethylxanthine (Sigma), 60 μmol/l indomethacin (Sigma), 10 μg/ml insulin (Sigma) and 1 μmol/l desamethasone. After 2 weeks of induction, the cells were fixed and stained with oil red O solution.

Neural differentiation. Adherent cells were induced according to Woodbury *et al* (2000) with some modification. Cells were seeded after using attachin (bio999, Taichung, Taiwan), cells were pretreated overnight in DMEM plus 20% FBS and 10 ng/ml bFGF, then medium was removed and replaced with induction medium, composed of DMEM plus 1 × N2 medium (Gibco BRL), 2% FBS, 2% dimethyl sulphoxide (DMSO; Sigma) and 200 μmol/l butylated hydroxyanisole (BHA; Sigma). After 10 h, cells were fixed and stained for a series of neural cells markers, including β-tubulin III, glial fibrillary acidic protein (GFAP), microtubule-associated protein 2a,b (MAP2a,b), Nestin (NSE) and neurofilament M (NF-M, 145 kDa). Another induction method used DMEM plus 5% FBS, 1 × N2 medium and 50 ng/ml bFGF; after 7 d, the

Table I. Primer sets.

Gene	Primer sequence	Product size (bp)
NSE	Forward: 5′-TTACTTAGGCAAAGGTGTCCTGAA-3′ Reverse: 5′-GTTTCTCTTGCTCCACCACAG-3′	101
MAP2	Forward: 5′-AATAGACCTAAGCCATGTGACATCC-3′ Reverse: 5′-AGAACCAACTTTAGCTTGGGCC-3′	133
β-tubulin III	Forward: 5′-CATGGACAGTGTCCGCTCAG-3′ Reverse: 5′-CAGGCAGTCGCAGTTTTCAC-3′	175
GFAP	Forward: 5′-CTGGAGGTTGAGAGGGACAATCT-3′ Reverse: 5′-TACTGCGTGCGGATCTCTTTC-3′	317
Oct4	Forward: 5′-GACAACAATGAAAATCTTCAGGAGA-3′ Reverse: 5′-TTCTGGCGCCGGTTACAGAACCA-3′	218
Nestin	Forward: 5′-GCCCTGACCACTCCAGTTTA-3′ Reverse: 5′-GGAGTCCTGGATTTCCTTCC-3′	200
Bcrp1	Forward: 5′-GGCCTCAGGAAGACTTATGT-3′ Reverse: 5′-AAGGAGGTGGTGTAGCTGAT-3′	342
β-actin	Forward: 5′-GTGGGCATGGGTCAGAAG-3′ Reverse: 5′-GAGGCGTACAGGGATAGCAC-3′	302

culture medium was replaced by DMEM plus 5% FBS, 1 × N2 medium, 10 ng/ml brain-derived neurotrophic factor (BDNF; PeproTech EC Ltd, London, UK) and 10 μmol/l retinoic acid (Sigma) and cultured for a further 7 d.

Immunofluorescence

For staining of cell membrane proteins, cells were rinsed with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min, then rinsed and incubated with the FITC-labelled anti-human CD45 mouse monoclonal antibody. For staining of cytoskeletal proteins, cells were rinsed, fixed and permeabilized with 0·1% Triton X-100 (Sigma) for 15 min, then blocked using normal goat serum for 30 min. Cells were incubated overnight at 4°C with primary antibodies, including mouse monoclonal antibodies against β-tubulin III (1:100; Chemicon), Nestin (1:200; Chemicon), MAP2a,b (1:100; Neomarkers, Fremont, CA, USA), rabbit polyclonal antibodies against GFAP (1:100; Chemicon) and neurofilament medium (NF-M, 1:200; Chemicon). Then cells were rinsed and incubated with either etramethyl rhodamine isothiocyanate (TRITC)-labelled goat anti-mouse IgG or FITC-labelled goat anti-rabbit IgG antibodies for different primary antibodies. After 1 h at room temperature, cells were rinsed and observed under an Olympus fluorescent microscope. Cell nuclei were counterstained with Hochest 33342 (Sigma).

Reverse transcription–polymerase chain reaction (RT-PCR) analysis

Total RNA, from CFU-FC or neuronally differentiating cells, was isolated with trizol reagent. cDNA was synthesized using the Moloney murine leukaemia virus (MoMLV-H) reverse transcriptase system (Gibco BRL). cDNA was used as template for PCR amplification in a 25-μl reaction volume using gene-specific primer pairs designed using sequences obtained from the GenBank database. PCR products were amplified over 30–35 cycles (denaturing for 30 s at 94°C, annealing for 30 s according to optimal annealing temperature of different primers, extension for 45 s at 72°C). Primer sets were as listed in Table I.

Results

Characteristics of cultured blood adherent cells

Nineteen full-term UCB harvests and nine preterm fetal blood samples were cultured in this study; the results are shown in Table II.

Ninety per cent of full-term UCB harvests ($n = 17$) formed an adherent cell layer in 2 weeks; most of the derived adherent cells ($n = 15$) morphology was heterogeneous, approximately five types of cells could be identified: (i) large, round-shaped cells with thin cytoplasmic extensions and smooth borders; (ii) multinucleated giant cells; (iii) scattered fibroblast-like cells, some with a splayed cap at the end or short dendritic processes; (iv) fibroblast-like cells forming a cord-like structure; and (v) small, round cells with a large nucleus (Fig 1).

Table II. Characteristics of cultured blood adherent cells.

	Sample	A	B	C
Term UCB	19	2	15	2
Preterm fetal blood	9	8	0	1
Total number	28	10	15	3

A, adherent cells containing fibroblast colony-forming units (CFU-Fs); B, heterogeneous adherent cells without CFU-Fs; C, rare or no adherent cells; UCB, umbilical cord blood.

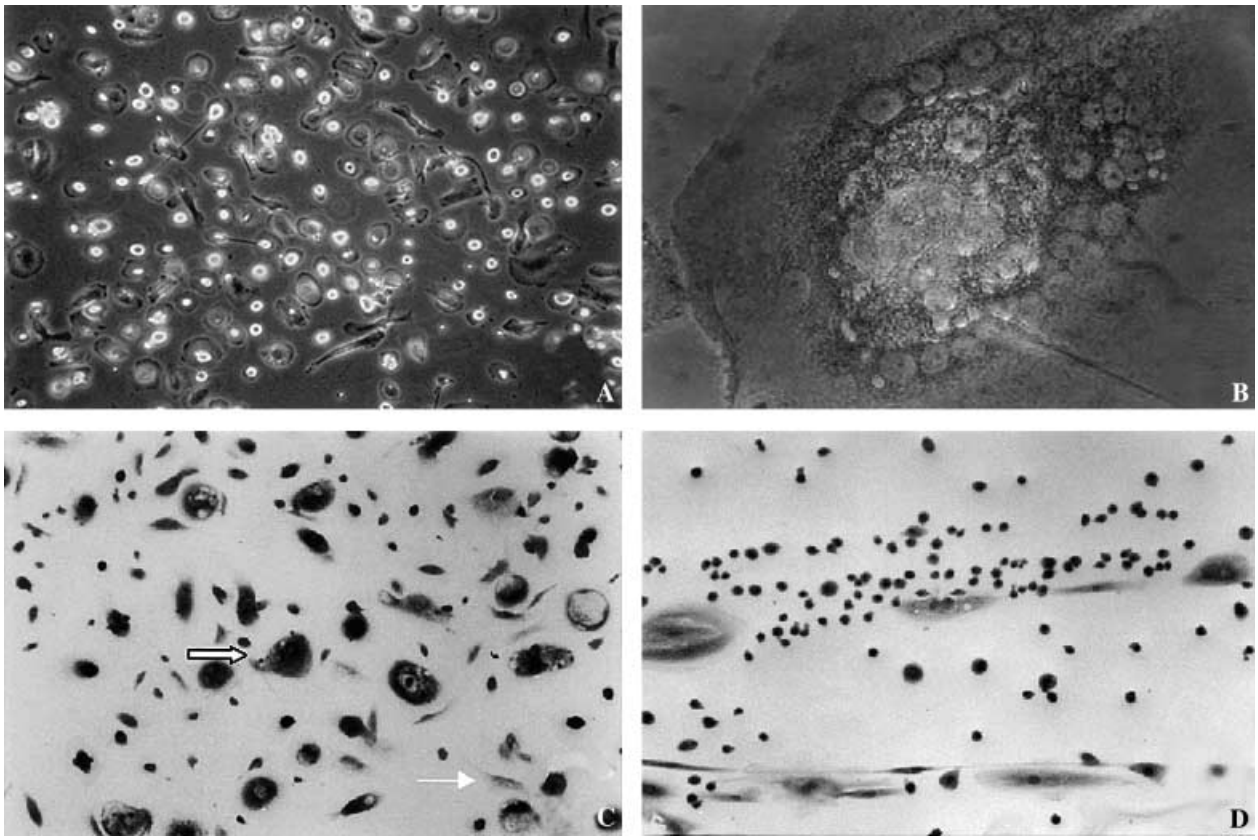

Fig 1. (A) UCB-derived adherent heterogeneous cell population (×100 magnification); (B) multinucleated giant cells (×400 magnification); (C) oil red O staining of heterogeneous cell population (×200 magnification) with haematoxylin-stained nucleus; note the large, round shaped cells containing lipid droplets stained by oil red O (wide arrow). Fibroblast-like cells did not contain lipid droplets (thin arrow). (D) Fibroblast-like cells with a cord-like structure. Some small, round cells with a large nucleus are also observed (haematoxylin stain, ×100 magnification).

No obvious CFU-Fs were observed in these samples. They reached confluence after about 2 weeks. Large, round-shaped cells and fibroblast-like cells were prevalent. Sometimes they formed obvious heterogeneous cell clusters as early as day 3. Multinucleated giant cells occurred around day 12. Although heterogeneous adherent cells could reach confluence in primary passage, their proliferation capacity was limited. They were never passaged beyond the second passage.

Eight of nine fetal blood samples (gestational age 16–34 weeks) and two full-term UCB samples contained homogeneous CFU-F. Obvious cell clones could be observed at about 14 d. They adhered to the bottom of the culture flask, proliferated very quickly and gained superiority over other cells, forming a homogeneous cell layer in 3 weeks. The amounts varied to a great extent. The frequency of adherent colonies declined with increasing fetal age and varied from 0 to $2{\cdot}5/10^6$ MNCs (Fig 2). These cells could be passaged and maintained *in vitro* for more than 4 months.

Immunophenotype of cultured adherent cells

The immunophenotype of heterogeneous cells was determined by FACS and immunofluorescence techniques. The majority of cells were $CD45^+$, some were $CD14^+$, indicating their haematopoietic origin and monocyte/macrophage phenotype (Fig 3).

The CFU-FC surface markers were $CD14^-$, $CD45^-$, $CD34^-$, $HLA\text{-}DR^-$, with high-level expression of CD44, CD90 and low-level expression of CD71, CD105; the phenotype of two cell clones was also similar. Bcrp1, a molecular determinant of the side-population phenotype (Zhou *et al*, 2001), and Oct4, an important transcription factor of pluripotent stem cells (Nichols *et al*, 1998), were both expressed on CFU-FC (Fig 4).

Proliferation studies and cell cycle analysis

CFU-FC could be maintained over 4 months and beyond 20 passages. Cell growth curve was depicted through cell count and conversion at each passage (Fig 5A; $n = 1$, gestational age = 20 weeks). The mean cumulative number of population doublings was 52·15 in 124 d. The mean cell population doubling time was 57·07 h. Cell cycle analysis (at log phase of growth) was performed on fifth-passage CFU-FC (Fig 5B; $n = 3$). On average, 72·8% cells were in G0–G1 phase and 14·11% cells were in S phase. Single cell clone seeding was also performed on the fifth passage. Their colony-forming efficiency was 27·7%. We selected 11 clones randomly and

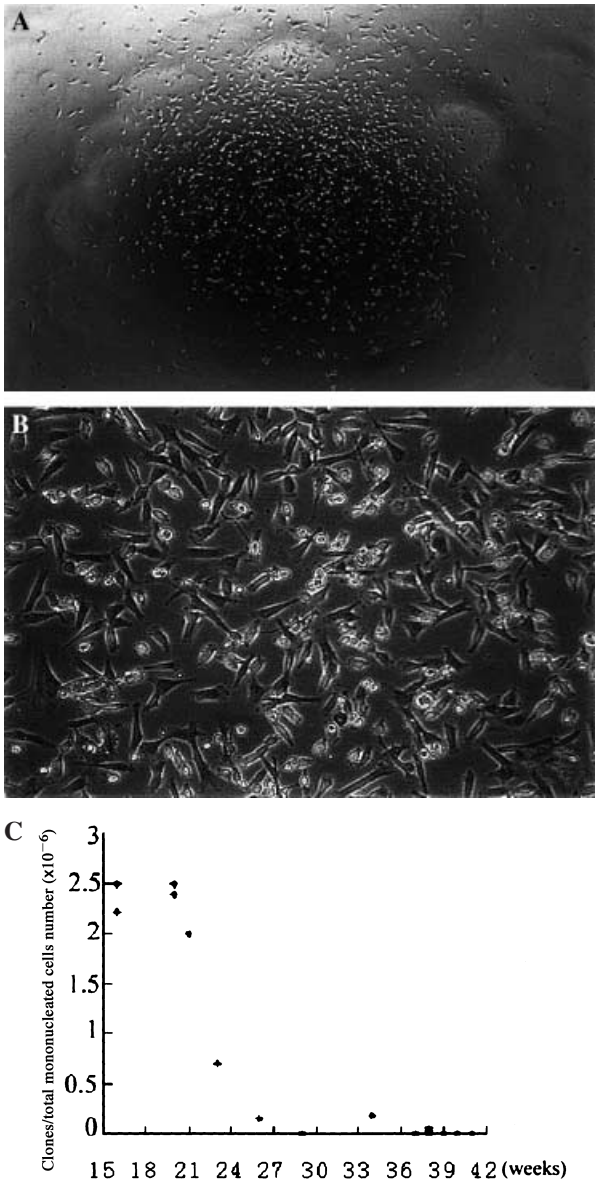

Fig 2. (A, B) A CFU-F clone derived from the blood of a 20-week-old fetus (A, ×40 magnification; B, ×100 magnification). (C) CFU-F clone colony-forming efficiency in fetal blood-cultured samples.

recorded their growth curve (Fig 5C; $n = 11$). The mean cumulative number of population doublings was 23·08 ± 4·15 in 34 d. The mean cell population doubling time was 35·36 ± 7·75 h.

Adherent cells differentiation

Osteogenic and adipogenic differentiation. The differentiating ability of blood CFU-FC was tested under conditions that favoured either adipogenic, osteogenic or neural differentiation. After 21 d under osteogenic differentiation conditions, about 80% of cells were ALP positive; ALP positive cells were rare under ordinary culture conditions. Extensive

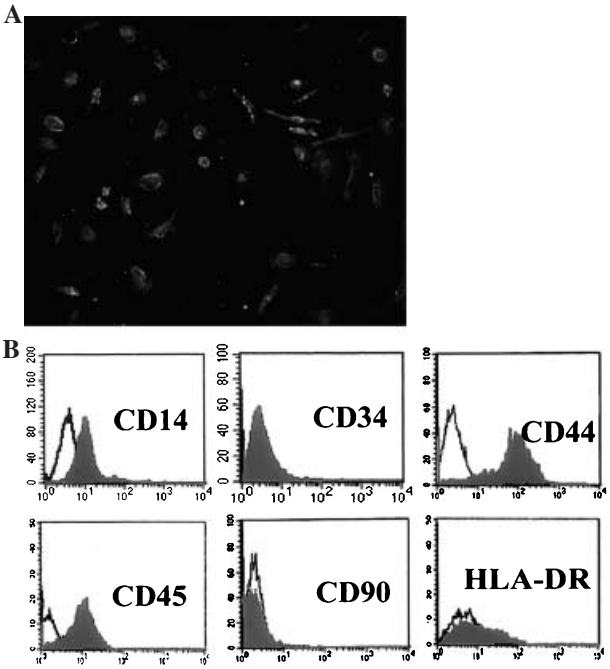

Fig 3. (A) Most heterogeneous cell populations derived from postnatal infants' UCB were $CD45^+$. Note that the large round cells and fibroblast-like cells were all $CD45^+$ (×200 magnification). (B) The immunophenotype of the heterogeneous cell population was analysed by FACS.

mineralization occurred throughout osteogenic cultures, but never in ordinary conditions. Under adipogenic differentiation conditions, the morphology of some cells changed, from a slim, spindle shape to a cuboid shape. Lipid vacuoles surrounding the nucleus were obviously visible after 6 d of induction; 14 d after induction, differentiated cells were identified by lipid vacuoles stained by oil red O solution. More than 95% of cells contained lipid vacuoles in comparison with control cells, which stained negative by oil red O. Clone 5F6, that underwent over 23 doublings after single cell seeding was also tested for osteogenic and adipogenic differentiation. There was no obvious difference in calcium deposition, but the adipogenic differentiation of clone-derived cells was significantly diminished. Only about 50% of clone-derived cells contained lipid droplets stained by oil red O. The percentage of the lipid droplets area was also significantly reduced when compared with cell plasma. Interestingly, the surface area of these clone-derived cells was remarkably expanded compared with the primary passage CFU-FC (Fig 6). In comparison, we failed to induce UCB-derived heterogeneous cells to differentiate into osteoblasts and adipocytes because most of the cells died during the induction period.

Neural differentiation. Under bFGF, DMSO and BHA treatment, about 60% of CFU-FC showed contraction of the cell bodies and the emergence of processes after 10 h of

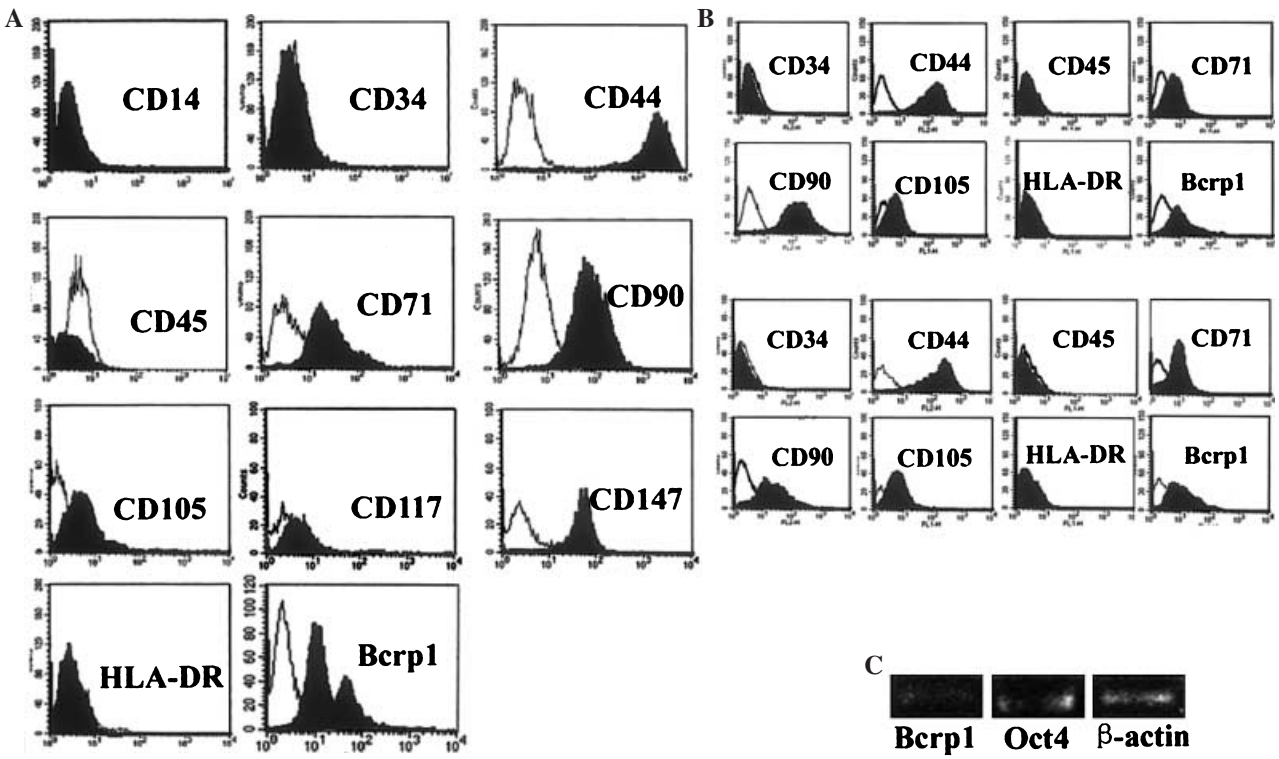

Fig 4. (A) Immunophenotype of cultured CFU-FC. (B) Two CFU-FC clones also showed similar immunophenotype. (C) RT-PCR showed Bcrp1 and Oct4 expression of CFU-FCs.

induction. Immunofluorescence showed some neuron-like cells began to express β-tubulin III and MAP2 (Fig 7A and B). Eighty per cent of cells were Nestin positive (Fig 7C). No cells expressed GFAP (Fig 7D), but under bFGF and BDNF treatment about 15% of cells began to express GFAP (Fig 7E). Two colonies also shared similar differentiation ability (data not shown). RT-PCR analysis of neural cell marker expression after DMSO and BHA treatment showed increased NSE expression after induction. Cells began to express MAP2 and β-tubulin III after induction. The expression of the neural progenitor cell marker, Nestin, did not change remarkably after induction. Cells did not express astrocytes marker GFAP after induction. β-Actin served as the loading control (Fig 7H).

Discussion

In this study, we have described the different characteristics of adherent cells in preterm fetal blood and full-term UCB. Most of the term UCB-derived adherent cells were heterogeneous, and they displayed different morphology. Most of the cells were $CD45^+$, which indicated they were derived from haematopoietic cells; some were $CD14^+$, which hinted at their monocyte/macrophage derivation; $CD90^-$, which ruled out the possibility of mesenchymal origin. Large, round cells with thin cytoplasmic extensions and scattered fibroblast-like cells were all derived from monocytes as they were $CD14^+$ (Wilkins & Jones, 1995; Gutierrez-Rodriguez *et al*, 2000; Zhao *et al*, 2003). It was unusual that a high proportion of giant multinucleated cells occurred after 2 weeks, and they were identified as osteoclast-like cells, as previously reported (Erices *et al*, 2000). They were thought to derive from monocyte/macrophage precursor cells. Macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand are critical differentiation factors of osteoclasts (Nakagawa *et al*, 1998; Miyamoto *et al*, 2001). But why did osteoclast-like cells occur in our culture system without any cytokine supplements? We suspect that this may be due to cell–cell contact interaction after confluence because no osteoclast-like cells were observed prior to culture or culture in low cell density. Some fibroblast-like cells were thought to be endothelial cells because they formed a cord-like structure in the appropriate conditions (Milici *et al*, 1985). The presence of endothelial precursor cells in UCB has been reported (Ye *et al*, 1994; Nieda *et al*, 1997), but other reports showed that endothelial cells could also be differentiated from monocytes/macrophages (Schmeisser *et al*, 2001; Havemann *et al*, 2003; Rehman *et al*, 2003). Further research should be undertaken to clearly demonstrate the origin of the heterogeneous cell population and the evolutionary relationship among them.

In contrast, preterm, mainly mid-trimester, fetal blood (16–26 weeks) enhanced CFU-F formation. Obvious CFU-F colonies were seen to occur within 14 d, they proliferated rapidly and became prevalent over other cells in a short time. According to our data (Fig 3), we assumed that their

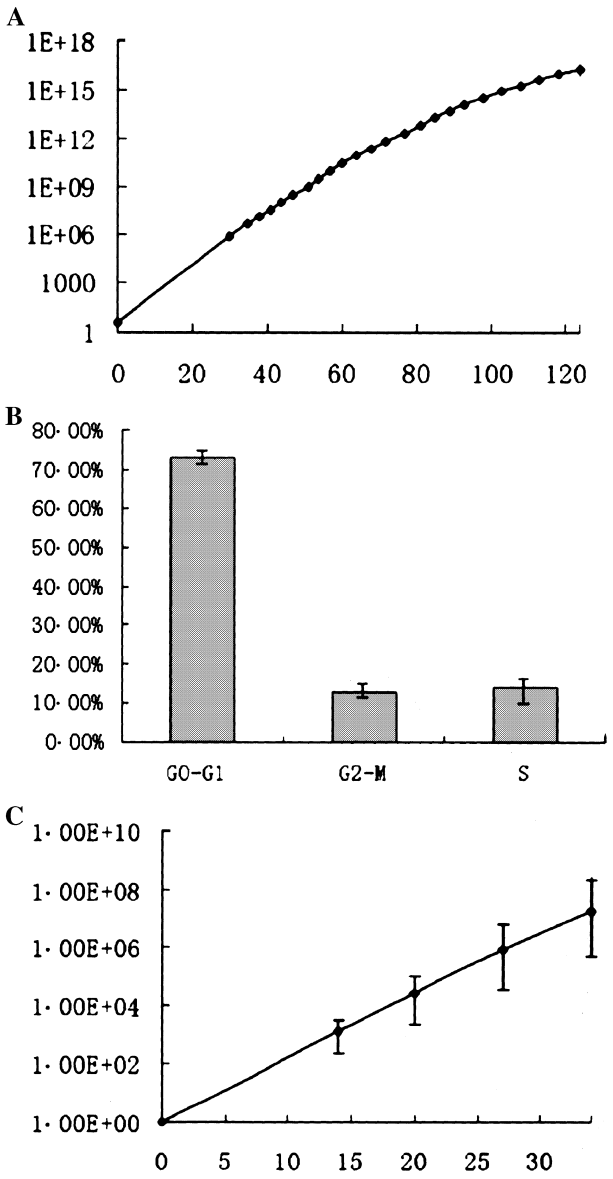

Fig 5. (A) Growth curve of CFU-FCs. Abscissa: days of culture, ordinate: cell number ($n = 1$). (B) Cell cycle analysis of CFU-FCs ($n = 3$). Abscissa: three cell phases, ordinate: percentage of cells in each phase. (C) Growth curve of 11 colonies. Abscissa: days of culture, ordinate: cell number. Results are expressed as mean ± standard error of the mean (SEM).

frequencies decreased as gestational age increased, in accordance with a previous report (Erices *et al*, 2000). The phenotype of the CFU-FC was, in accordance with a previous report (Pittenger *et al*, 1999), like BMMSCs. CD105 (endoglin) was expressed by CFU-FC and thought to be a putative MSCs surface marker (Barry *et al*, 1999; Zvaifler *et al*, 2000). They expressed Oct4 and Bcrp1, which indicated pluripotent stem cell characteristics. Their proliferation rate was fast and they could be passaged beyond the 20th passage. Their differentiation ability was tested under osteogenic, adipogenic and neural differentiation conditions. Our results show they shared similar characteristics to BMMSCs. Single colonies were also tested for the differentiation of three lineages after undergoing more than 23 doublings. Our results indicate no obvious difference in osteogenic and neuronal differentiation, but the adipogenic differentiation potential of the colonies was significantly diminished. This was probably because the cells gradually lost their extensive differentiation potential after undergoing many cycles of cell division (Digirolamo *et al*, 1999; Colter *et al*, 2001).

There are several explanations for the different cell types that were derived from UCB and preterm fetal blood. First, the MSCs could migrate, along with haematopoietic cells, from the early haematopoietic site to the bone marrow. This migration is almost complete in full-term newborn babies, which explains why CFU-F are rare in UCB and adult peripheral blood. Another possibility is that when haematopoietic cells, particularly immunocytes such as lymphocytes and macrophages, gradually mature, or other accessory cells occur with gestational age, they inhibit the proliferation of CFU-F. Thirdly, the CFU-Fs in full-term UCB did not proliferate in our culture system. However, this is not a satisfactory explanation unless there are significant differences between full-term and early fetal blood CFU-F.

Although we could not effectively separate any type of pluripotent stem cell from newborn UCB, this does not exclude that other types of stem cells reside in postnatal circulation blood. Some reports showed a form of neural progenitor-like cells could be effectively separated from mouse peripheral blood (Torrente *et al*, 2002) and human UCB (Sanchez-Ramos *et al*, 2001; Buzanska *et al*, 2002); these cells were Nestin positive, like neural progenitor cells, and they could be induced to differentiate into neurons and glia. Buzanska *et al* (2002) showed that these cells were $CD34^-$ $CD45^-$, which excluded a haematopoietic origin. Labat *et al* (2000) showed neural crest origin monocytoid cells also subsist in normal human peripheral blood. These cells shared similar characteristics to MSCs. They could adhere to the culture flask and expressed a series of neural markers, such as neurofilament 160 (Labat *et al*, 2000). Zhao *et al* (2003) reported that peripheral blood-derived monocytes with the $CD45^+$ $CD14^+$ immunophenotype, could also be induced to differentiate into other non-haematopoietic cell types including epithelial, endothelial, neuronal and liver cells. We also separated heterogeneous cell populations, such as standard macrophages (s-Mϕ) and fibroblast macrophages (f-Mϕ), as described by Zhao *et al* (2003). Some s-Mϕ also contained lipid droplets. However, we were unable to make these cells proliferate, as we did not add any essential cytokines, such as M-CSF, to the culture medium.

In conclusion, there are obvious differences in the composition of adherent cells that have been cultured from different gestational age fetal blood. Mesenchymal stem-like cells mainly subsist in fetal blood with a gestational age of 16–26 weeks or earlier.

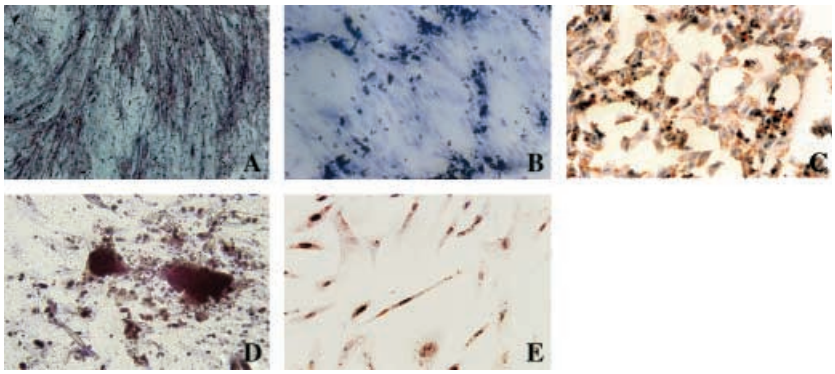

Fig 6. Osteogenic and adipogenic differentiation of CFU-FC. Osteogenic differentiation of CFU-FC (A, B, D). Osteogenic differentiation was indicated by alkaline phosphatase staining (A, ×100 magnification) and calcium deposition was stained using the von Kossa method (B, ×100 magnification; D, cell clone 5F6, ×200 magnification). Adipogenic differentiation was indicated by oil red O staining (C, ×200 magnification; E, cell clone 5F6, ×200 magnification).

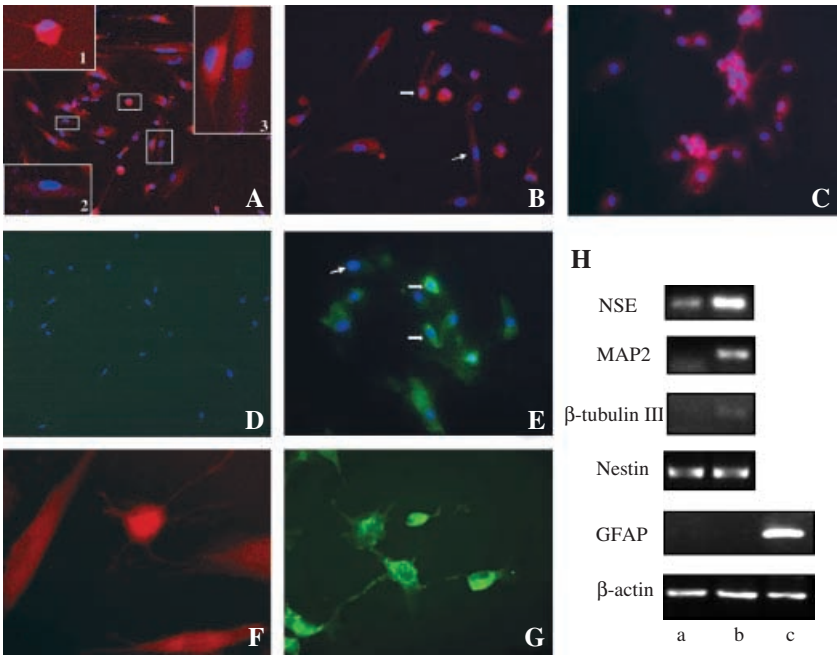

Fig 7. Neural differentiation of CFU-FC. (A–E) merged images, cell nucleus counterstained by Hochest 33342. Neuronal induction medium-treated cells were stained by mouse monoclonal antibodies against human MAP2a,b (A, ×100 magnification); β-tubulin III (B, ×200 magnification); Nestin (C, ×200 magnification) and rabbit polyclonal antibody against human GFAP (D, ×100 magnification). Note that cells with contracted cell body and long processes expressed much higher levels of MAP2 compared with fibroblast-like cells (A1–3). One cell with a round body and long process was β-tubulin III positive (B, wide arrow), but another fibroblast-like cell was not (B, thin arrow). Cells under bFGF, BDNF and retinoic acid treatment began to express GFAP (E, ×200 magnification). Two GFAP positive cells (wide arrow) and one GFAP negative cell (thin arrow) are shown in Fig 7E. A cell with long processes was MAP2 positive (F, ×400 magnification) and NF-M positive (G, ×200 magnification). (H) RT-PCR analysis of neuronal induction using DMSO plus BHA. (a) Pre-induction, (b) after induction and (c) positive control, neural progenitor cells derived from fetal hippocampus.

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